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The use of dried blood spots for quantification of 15 antipsychotics and 7 metabolites with ultra-high performance liquid chromatography – tandem mass spectrometry

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Abbreviations

7-hydroxy-N-desalkyl-quetiapine, 7OH-NDA-QUE; 7-hydroxy-quetiapine, 7OH-QUE; amisulpride, AMI; antipsychotic, AP; aripiprazole, ARI; asenapine, ASE; bromperidol, BRO; clozapine, CLO; dehydro-aripiprazole, DARI; dried blood spot, DBS; dynamic multiple-reaction monitoring, dMRM; electrospray ionisation, ESI; haloperidol, HAL; hematocrit, HCT; hydroxy-iloperidone, HILO; iloperidone, ILO; internal standard, IS; levosulpiride, LSUL; liquid chromatography, LC; lower limit of quantitation, LLOQ; lurasidone, LUR; methyl *tert*-butyl ether, MTBE; mass spectrometry, MS; multiple-reaction monitoring, MRM; N-demethyl-clozapine, NDM-CLO; N-demethyl-olanzapine, NDM-OLA; olanzapine, OLA; paliperidone, PAL; pipamperone, PIP; quetiapine, QUE; reduced haloperidol, RHAL; retention time, RT; risperidone, RIS; sertindole, SER; stable isotope labeled internal standard, SIL-IS; therapeutic drug monitoring, TDM; ultra-high performance liquid chromatography-tandem mass spectrometry, UHPLC-MS/MS; zuclopenthixol, ZUC;

1. Introduction

Antipsychotics (APs) are used for treatment of psychotic symptoms in patients with schizophrenic, schizophreniform, schizoaffective, psycho-organic and bipolar disorders [1-4]. A combination of psychotherapy and pharmacotherapy can improve symptoms significantly. However, monitoring of these APs in serum or plasma is often recommended. Therapeutic drug monitoring (TDM) can aid in finding the right therapy, explaining non-response, pharmacokinetic interactions or poor response [5, 6].

Especially in psychiatric populations, classical venous blood sampling is often experienced as unpleasant and even frightening. The interest in alternative sampling techniques, like dried blood spots (DBS), has consequently increased. DBS sampling is a micro-sampling technique where a drop of capillary blood is spotted on special filter paper. This technique has been used routinely since the 1960s, when Guthrie described a method to detect phenylketonuria in newborns [7]. DBS sampling has a lot of advantages, including ease of sampling, less invasive and inexpensive sampling, transport and storage [8, 9]. Since only very small volumes of blood are collected (typically between 10 and 80 µl), interest in DBS methods has increased in the last two decades due to the availability of more sensitive analytical techniques [8, 10, 11].

Interest in DBS sampling for TDM has recently increased [11, 12]. DBS methods have been described for monitoring of e.g. antidepressants, antiretroviral drugs, antibiotics, antiepileptic drugs, chemotherapeutic agents, antimycotics and immunosuppressants [12]. Some DBS methods for multiple drugs including one or two APs are already published [13-15]. No analytical method for determination of multiple AP using DBS has been reported yet.

Analyzing multiple APs in one method makes it possible to monitor polymedicated patients and to analyze clinical samples of different patients containing any of these APs in one batch. We aimed to develop a fast and easy to perform DBS method for quantification of 16 APs and 8 metabolites using a highly sensitive ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) technique. Selection of these 16 APs was based on their importance on the worldwide market and includes the newer APs like asenapine, iloperidone and lurasidone. Metabolites showing pharmacological activity or helping in interpretation of TDM data were also selected. Since paliperidone (9-hydroxy-risperidone) is both a parent compound and a metabolite of risperidone, the total amount of compounds included in the method is 23. Except for bromperidol and levosulpiride, deuterated internal standard (IS) were used for each individual compound [16].

2. Experimental

2.1 Chemicals and reagents

7-hydroxy-N-desalkyl-quetiapine dihydrochloride (7OH-NDA-QUE), 7-hydroxy-quetiapine (7OH-QUE), amisulpride (AMI), aripiprazole (ARI), asenapine (ASE), bromperidol (BRO), clozapine (CLO), dehydro-aripiprazole hydrochloride (DARI), haloperidol (HAL), hydroxy-iloperidone (HILO), iloperidone (ILO), levosulpiride (LSUL), lurasidone hydrochloride (LUR), N-demethyl-clozapine (NDM-CLO), N-demethyl-olanzapine (NDM-OLA), olanzapine (OLA), paliperidone (PAL), pipamperone dihydrochloride (PIP), quetiapine hemifumarate (QUE), reduced haloperidol (RHAL), risperidone (RIS), sertindole (SER) and zuclopenthixol succinate salt (ZUC) were purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). The stable isotope labelled internal standards (SIL-IS) 7OH-NDA-QUE-d_8 dihydrochloride, 7OH-QUE-d_8, AMI-d_5, ARI-d_5, ASE-$^{13}C,d_3$, CLO-d_8, DARI-d_8 hydrochloride, HAL-d_4, HILO-d_4,

ILO-d_3, LUR-d_8 hydrochloride, NDM-CLO-d_8, NDM-OLA-d_8, OLA-d_8, PAL-d_4, PIP-d_{10} dihydrochloride, QUE-d_8 fumarate, RHAL-d_4, RIS-d_4, SER-d_4 and ZUC-d_4 succinate salt were also purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Acetonitrile, acetic acid, formic acid, methanol and methyl *tert*-butyl ether (ethanol stabilized) (MTBE) were from Merck (Darmstadt, Germany). All chemicals were of LC quality.

2.2 Standards

Methanolic stock solutions of 7OH-NDA-QUE, 7OH-QUE, AMI, ASE, BRO, HAL, RHAL, LUR, LSUL, PIP, QUE, SER and ZUC were prepared at a concentration of 1 mg/ml. ARI, CLO, DARI, HILO, ILO, NDM-CLO, NDM-OLA, OLA, PAL and RIS stock solutions were prepared in acetonitrile at a concentration of 1 mg/ml. Working solutions of each analyte (100, 10 and 1 µg/ml) were prepared by further dilution of the stock solutions with acetonitrile. Methanolic stock solutions of 7OH-NDA-QUE-d_8, 7OH-QUE-d_8, AMI-d_5, ASE-$^{13}C,d_3$, HAL-d_4, RHAL-d_4, LUR-d_8, PIP-d_{10}, QUE-d_8, SER-d_4 and ZUC-d_4 were prepared at a concentration of 100 µg/ml. ARI-d_8, CLO-d_8, DARI-d_8, HILO-d_4, ILO-d_3, NDM-CLO-d_8, NDM-OLA-d_8, OLA-d_8, PAL-d_4 and RIS-d_4 stock solutions were prepared in acetonitrile at a concentration of 100 µg/ml. A working solution containing a mixture of all SIL-IS was prepared in acetonitrile by dilution of the stock solutions. The final concentration of the deuterated compounds ranged between 5 and 150 ng/ml, i.e. in the range of calibration level 3 or level 4 of the non-deuterated compounds.

The calibration standards consisted of a mixture of the working solutions containing the 23 analytes at 7 concentration levels. The chosen calibration ranges cover both the defined therapeutic ranges and the supratherapeutic concentrations [5]. The quality control (QC) standards were also prepared as a mixture from the different working solutions at 3

concentration levels (QC low, QC mid and QC high). All solutions were stored at -20°C. Twenty µl of the calibration and QC standards were spiked to 180 µl of human whole blood to yield final concentrations as shown in table 1. Although we realize that addition of 10 % organic solvent to blood is suboptimal and may affect blood properties to some extent, this was not problematic as our procedure utilizes complete spots for analysis.

2.3 DBS sample collection

As known, the hematocrit (HCT) is identified as the single most important parameter influencing the spread of blood on DBS cards, affecting the spot formation, spot size, drying time, homogeneity, the robustness and reproducibility of these assays [8, 17, 18]. In order to overcome this problem, it was decided to analyze the entire spot instead of using discs generated by punching only a part of the DBS. Hence, all issues concerning the spreading of blood could be avoided [19]. However, the influence of the HCT on other parameters, like matrix effects (ME), remained to be investigated [8].

Patient DBS samples were collected as follows: the preferred finger was disinfected with a 70% isopropanol cloth, air-dried and warmed for a few minutes. With the help of a single-use automatic lancet (Accu-Chek® Safe-T-Pro Plus, Roche Diagnostics, Mannheim, Germany), the fingertip was pricked. The first drop of blood was wiped off, since it contains an important amount of tissue fluid [12]. The second drop was collected in a 25-µl precision capillary 'end to end' (Hirschmann Laborgeräte, Eberstadt, Germany). Once entirely filled, the capillary was placed in the center of a marked circle on FTA™ DMPK-C Cards (GE Healthcare, Freiburg, Germany) until the capillary was completely emptied.

2.4 Sample preparation and extraction

Twenty-five µl of whole blood was spotted onto DMPK-C cards. In line with commonly applied drying times described in literature [12, 19], these were left to dry for at least 3h at room temperature. The whole DBS was excised and collected into a 2-ml Eppendorf tube (Eppendorf AG, Hamburg, Germany). Extraction was performed by adding 450 µl of methanol, 150 µl of methyl *tert*-butyl ether (MTBE) and 12.5 µl of the IS working solution. After shaking the samples during 5 min on an Eppendorf MixMate (Eppendorf AG), the extract was transferred to a 1.5-ml Eppendorf tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. The samples were reconstituted in 50 µl of aqueous ammonium acetate (10 mM)/acetonitrile (9:1; v/v), vortexed for 30 s and centrifuged for 2 min at 10 000 x *g*. After transferring the extracts to an autosampler vial, a volume of 10 µl was injected into the UHPLC-MS/MS system.

2.5 Instrumentation and analytical method

Samples were analyzed on an Agilent 1290 Infinity LC system (Agilent Technologies, Santa-Clara, California, U.S.A.) coupled with an Agilent 6460 Triple Quadrupole mass spectrometer (MS) run in Jetstream® electrospray ionization (ESI) mode. Data were acquired and analyzed with Masshunter Workstation Software version B.06.00 (Agilent Technologies). The LC system was optimized for rapid resolution.

Separation was achieved using an Agilent SB C_{18} reversed phase column (2.1 x 50 mm, 1.7 µm) (Agilent Technologies) with column oven temperature at 40°C. Gradient elution was performed at a flow rate of 0.5 ml/min with 10 mM aqueous ammonium acetate at pH 3.7 (A) and acetonitrile (B) using the following program: starting conditions 10 % B; increase to 75 % B between 0 and 2.5 min; further increase to 95 % B between 2.5 and 3 min; retain 95% B between 3 and 4.5 min; go back to initial conditions from 4.6 to 6.

The MS conditions were optimized as follows: Jetstream ESI technology, positive mode, nebulizer gas: nitrogen, sheat gas temperature: 400°C, sheat gas flow: 12 L/min, nebulizer pressure: 50 psi, capillary voltage: 3000 V, and nozzle voltage: 0 V.

The MS was operated in dynamic multiple-reaction monitoring (dMRM) mode, monitoring 3 ion transitions for each analyte in their specific retention time (RT) window (RT ± 0.25 min). The mass spectrometric conditions for each analyte are identical those of a previous method for simultaneous determination of the same 16 APs and 8 metabolites in serum (Supplemental data table 1) [16].

2.6 Validation

The following validation parameters were investigated according to in-house guidelines, which are based upon the international guidelines of EMA (European Medicines Agency) and FDA (Food and Drug Administration)[20, 21], modified by specific recommendations for forensic and clinical toxicology [22, 23]: selectivity, linearity, precision, accuracy, recovery, matrix effects, stability and incurred sample reanalysis.

Selectivity was evaluated by analyzing blank blood from eight different sources, two zero samples (blank blood + mix of SIL-IS) and two samples spiked with only analytes and no SIL-IS. Carryover was tested by injecting the highest concentration of the calibration curve, followed by two blank injections and should not exceed 20 % of the lower limit of quantitation (LLOQ) (n=2).

A seven-point calibration curve was analyzed using IS-corrected areas on each of four consecutive days. At each of these four days, duplicates of QC samples at LLOQ, low, medium and high concentration levels were analyzed. Intra-, interday-precision and accuracy were determined using an ANOVA-calculation as described by Wille et al. [23] Accuracy and

precision data were acceptable when the % bias respectively % coefficient of variation (% CV) was lower than 15 % (20 % for LLOQ).

Extraction recovery (ER) and matrix effects (ME) were determined at two concentration levels (QC low and QC high) using whole blank blood from six volunteers, spiked before and after extraction according to the post-extraction addition technique as described by Matuszewski et al [24]. ME are calculated as the percent ratio of peak areas of the analytes spiked after extraction and the blood free solution prepared in acetonitrile. ME were also evaluated at low and high HCT (19.2 and 67.0 %, respectively). Relative ME were calculated as the percent ratio of the IS corrected peak areas of the analytes spiked after extraction and the blood free solution. ER is calculated as the percent ratio of the IS corrected peak areas of the analytes spiked before and after extraction. % CV of the relative ME should not exceed 15 %.

Stability was evaluated at QC low and QC high (n=3) after 1 day, 1 week and 1 month at different storage conditions (room temperature, 4°C and -18°C). DBS were stored in zip-closure bags with desiccant. Finally, stability of the processed samples on the autosampler was determined by analyzing the extracts of QC low and QC high (n=3) after 6 and 12 h, respectively. Concentrations of all stability samples were calculated based on the daily calibration curves. The concentration of the stability samples had to be within 90-110 % of the mean of that of the control samples, and the 90 % confidence interval (CI) of the stability samples had to be within 80-120 % of the mean concentration of the control samples [22, 23].

Incurred sample reanalysis was performed on DBS samples of 20 different patients with a time interval of 1 to 3 months between initial analysis and reanalysis. Data were acceptable

when the % difference between the results was within ± 20 % of their mean for two-thirds of the samples [20].

3. Results & discussion

3.1 Filter paper selection, extraction procedure and detection

Two types of filter paper were tested, Whatman 903 paper (GE Healthcare, Freiburg, Germany) and Whatman FTA™ DMPK-C Cards (GE Healthcare). Both filter papers are cellulose cards not containing protein denaturing agents, which are known to cause ME [18]. After extraction of samples on Whatman 903 paper, higher matrix effects were seen in comparison with the DMPK-C cards. Consequently, DMPK-C cards were selected for further method development.

Different extraction solvents were evaluated: methanol, acetonitrile and MTBE, as well as mixtures of these solvents: methanol: acetonitrile at 1:1 (v/v), 3:1 (v/v) and 1:3 (v/v) and methanol: MTBE at 1:1 (v/v), 3:1 (v/v) and 1:3 (v/v). In literature, methanol, acetonitrile or mixtures of both are preferable for extraction because they cause protein denaturation and precipitation [12, 25]. Acetonitrile would yield a higher recovery and less matrix effects than methanol [9]. MTBE was also tested since it was selected as optimal extraction solvent for our serum method [16]. Highest recoveries were achieved with a 600 µl methanol: MTBE (3:1, v/v). Water had to be avoided as extraction solvent, since it is known to cause stability problems for olanzapine [16, 26, 27] and it increases the interference from endogenous compounds [12, 25].

Chromatographic conditions and MS parameters were adopted from our serum method for quantification of APs [16]. Only the source parameters of the MS had to be reevaluated in

275 order to increase sensitivity of the MS. Since absolute amounts in DBS are lower than in
276 serum, optimal sensitivity of the detector is mandatory.
277
278 **3.2 Validation experiments**
279 Analysis of eight different blank samples revealed no interference from endogenous
280 compounds nor from filter paper components. The response was less than 20 % of the LLOQ
281 at the mass transitions of the APs and less than 5 % of the response of the IS. For zero
282 samples, the response of the IS was less than 20 % of LLOQ at the transitions of APs. The
283 method proved to be highly selective. No carryover was observed. Injecting blanks right after
284 the highest calibrators yielded signals lower than 20 % of the LLOQ for all compounds.
285 Linearity was evaluated by analyzing four calibration curves on four consecutive days.
286 Unweighted and 1/x weighted linear regression were statistically and visually evaluated.
287 Inclusion of the zero value in the 95 % CI of the y-intercept indicates absence of constant
288 error, and a correlation coefficient of 0.99 or higher was pursued. If a linear curve without
289 1/x weighting would be used, standard error estimation would be biased. For all compounds,
290 linear regression (1/x weighting) provided the best fit, with r^2 of 0.99 or higher for 16 of the
291 23 analytes and a zero value in the 95 % CI for all compounds except for NDM-OLA. In
292 comparison, unweighted linear regression resulted in an r^2 higher than 0.99 for only 9 of the
293 23 compounds and a zero value in the 95 % CI for only 20 of the 23 analytes.
294 Heteroscedastiscity was proven for all compounds by plotting residuals versus nominal
295 concentrations. Consequently, 1/x weighted linear regression was applied for all
296 compounds. Accuracy was evaluated using EMA criteria, which state that the back-calculated
297 concentration should be within 15 % of the nominal value (20 % at LLOQ). This was fulfilled
298 for all compounds, except for OLA and NDM-OLA and for PIP at LLOQ. All calibration curves

were linear in the proposed range, except for OLA and its metabolite NDM-OLA. These compounds were not detected at the lowest concentration of the calibration curve and back-calculated concentrations were often aberrant. The underlying cause likely is the instability of these compounds in aqueous medium, since the extract was reconstituted in aqueous ammonium acetate [16, 26, 27]. As a result, OLA and NDM-OLA were excluded from further analysis.

On the other hand, if a mean calibration curve is generated from all calibration curves, aberrancies are seen for LUR. As a consequence, daily calibration is necessary for LUR. The LLOQ, defined as the lowest concentration of the calibration curve, could be accepted for all analytes, except for PIP. This implicates that PIP concentrations lower than 50 ng/ml (a subtherapeutic concentration) cannot be quantified reliably.

Precision and accuracy were determined at four concentration levels (LLOQ, QC low, QC mid and QC high), analyzed in duplicate on four consecutive days. Accuracy (% bias), intraday precision (repeatability) and interday precision (intermediate precision) were calculated from data obtained with ANOVA-analysis (Table 2) [23]. Except for PIP at LLOQ and QC low and for LUR at QC low, all accuracy data were within the acceptance criteria (bias ≤ 15%, for LLOQ ≤ 20%). Both intraday and interday precision are acceptable when the CV (%) is lower than 15 % (20 % for LLOQ). Only for LUR, aberrancies were seen for both intraday and interday precision at QC low and QC mid. EMA criteria were fulfilled for all other compounds.

An overview of all ME and ER is given in Table 3. The ER (IS corrected) varied between 28.7 % for 7OH-NDA-QUE and 84.5 % for PIP, with a median ER of 66.4 % for all compounds. Due to the use of a more apolar extraction solvent, ER was limited but sufficiently high for this method. The absolute median ME was 66.1 % (range 8.8 to 100.4 %). Ion suppression is seen

when ME are below 100 %, ion enhancement when ME are above 100 %. For most compounds, a significant amount of ME is seen with the DBS method. However, these ME are almost completely compensated by the use of SIL-IS (Supplemental data Figure 1). When calculating the IS corrected ME, a median ME of 98.8 % (range 86.2 to 125.8 %) was obtained. For all compounds, CV (%) of the IS corrected ME was lower than 8.5 % and fulfilled the criteria (< 15 %). Besides the DBS, also filter paper may contribute to high ME. Moreover, both methanol and MTBE are known to extract endogenous blood lipids and induce a high ME too. Polar lipids like glycerophosphocholines are soluble in methanol, while non-polar lipids like cholesterol, cholesterol esters and triacylglycerols are soluble in MTBE. Presence of lipids in the extract can have an influence on sensitivity, selectivity and reproducibility of results. Lipids would be less extracted when using acetonitrile [9]. Consequently, high ME are expected when using a combination of both methanol and MTBE. However, when acetonitrile was used, lower recoveries were seen and ME were comparable with methanol.

ER and ME values can be influenced by the HCT. HCT levels are normally about 41-50 % for men and 36-44 % for women [8, 28]. Since ER and ME were tested on blood samples of six different volunteers, the HCT of these volunteers was determined and were found within this normal range. According to literature, a range of 28-67 % HCT would cover the majority of adult human blood samples [18]. Therefore, ME were also tested on samples with low (19.2 %) and high HCT (67.0 %). Adjustment of HCT was made by removing or adding plasma to a whole blood sample of one of the volunteers. No difference was seen between the calculated ME at low, normal and high HCT (Supplemental data Figure 2).

Stability experiments were conducted in a way that represents the actual storage conditions and handling of the samples during the study. Stability was evaluated when storing DBS

samples during 1 day, 1 week and 1 month at different storage temperatures (room temperature, 4°C and -18°C). Results were compared with samples analyzed directly after drying for 3 h at room temperature (Table 4). All compounds were stable during 1 month at the 3 storage temperatures. The stability studies revealed no difference between storage at room temperature, 4°C and -18°C.

Stability of the extracted samples was also evaluated. Extracted samples were measured after 6 and 12 h while residing in the autosampler at room temperature. The acceptance criteria were not fulfilled for DARI, HILO, LSUL, LUR and ZUC at QC low and for 7-OH-NDA-QUE and LUR at QC high. For 7-OH-NDA-QUE, LSUL and HILO, the deviation from the ratio of means could be accepted, while the 90 % CI did not meet the criteria. Only for LUR, both the deviation from the ratio of means and the 90 % CI did not meet the criteria. In Figure 1, a decrease in concentration is only seen for LUR at both QC low and QC high. After 12 h, the concentration of LUR was decreased with 32.3 % at QC low and 25.2 % at QC high. Since LUR is not stable in the processed sample, this can explain the aberrancies in linearity, precision and accuracy. The longer it takes for an extracted sample to be analyzed on the instrument, the lower the concentration of LUR will be.

Capillary DBS samples from 20 psychiatric patients were reanalyzed within 1 to 3 months after the initial analysis. These 20 capillary samples contained 52 different antipsychotics and metabolites. The criterium for incurred sample reanalysis was fulfilled, since the % difference between the results was within 20 % of their mean for all compounds in all samples, except for 7-OH-NDA-QUE.

3.3 Clinical application

Applicability of the DBS method was evaluated on samples, collected from patients with schizophrenia or bipolar disorder, within the framework of a large clinical study. Patients had to be in 'steady-state' condition (reached after 5-7 half-lives of the drug), which was translated as an unchanged dose of the antipsychotic in the last 7 days before sample collection. From every patient, we collected capillary DBS samples, as well as serum and whole blood just prior to the morning dose of the AP (trough or minimal concentration). In this study, overall, 10 of the antipsychotics and 6 of their metabolites could be quantified in the DBS samples. Figure 2 illustrates 4 chromatograms of capillary DBS samples from 4 patients treated with one or more AP(s). Patient A was treated with aripiprazole (20 mg/day), patient B with both amisulpride (400 mg/day) and clozapine (500 mg/day), patient C with risperidone I.V. (62.5 mg/2 ml every 14 days) and patient D was treated with amisulpride (200 mg/day) and quetiapine (1400 mg/day). As can be seen in Figure 2, all antipsychotics were found in the DBS samples from these patients. Table 5 shows data for 10 antipsychotics, quantified in serum, blood and DBS from 10 different patients. Concentrations found in blood and DBS are quite similar. For some compounds, concentrations in serum differ more, since they are influenced by the blood:plasma(serum) distribution of the antipsychotics. Obviously, a large dataset, with sufficient coverage of each of the antipsychotics, is required to confirm the correlation between the different matrices, together with the clinical interpretation of the results.

4. Conclusion

The UHPLC-MS/MS method for analysis of APs and metabolites using DBS overall meets the requirements of both FDA and EMA for 15 out of the 16 APs and 7 out of the 8 metabolites

selected [20, 21]. Only OLA and NDM-OLA were rejected from the method, likely owing to their instability in the aqueous reconstitution solvent. Since LUR was not stable in the extracted DBS sample, analysis by UHPLC-MS/MS must be performed as quickly as possible for this compound. The short run time of our method (6 min) is highly beneficial in this respect. This DBS method has high potential in TDM of APs and can be a valuable alternative to the classic venous blood withdrawal currently used for monitoring.

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Figure 1 : Stability of the extracted dried blood spot samples, spiked with QC low (A) and QC high (B), while residing on the autosampler during 12 h (n=4). Stability was calculated as deviation from fresh samples.

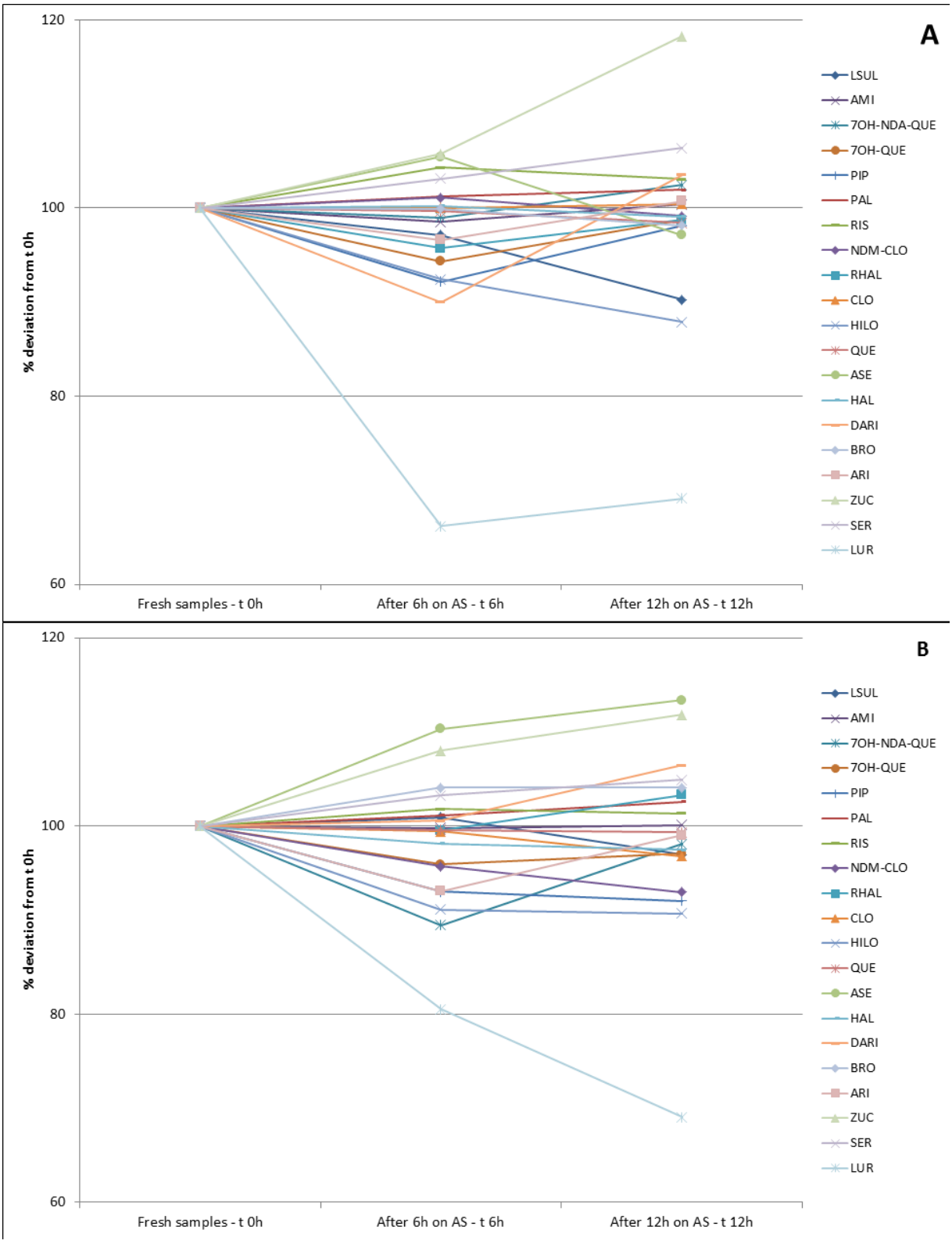


Figure 2: Representative chromatograms of capillary DBS samples obtained from 4 patients treated with multiple APs. MRM transitions of all antipsychotics and their deuterated IS are presented. (A) ARI and DARI (found concentrations were 184 and 180 ng/ml, respectively); (B) AMI, CLO and NDM-CLO (found concentrations were 270, 457 and 449 ng/ml, respectively); (C) PAL and RIS (found concentrations were 12 and 16 ng/ml, respectively); (D) AMI, QUE, 7OH-NDA-QUE and 7OH-QUE (found concentrations were 147, 152, 14 and 13 ng/ml, respectively).

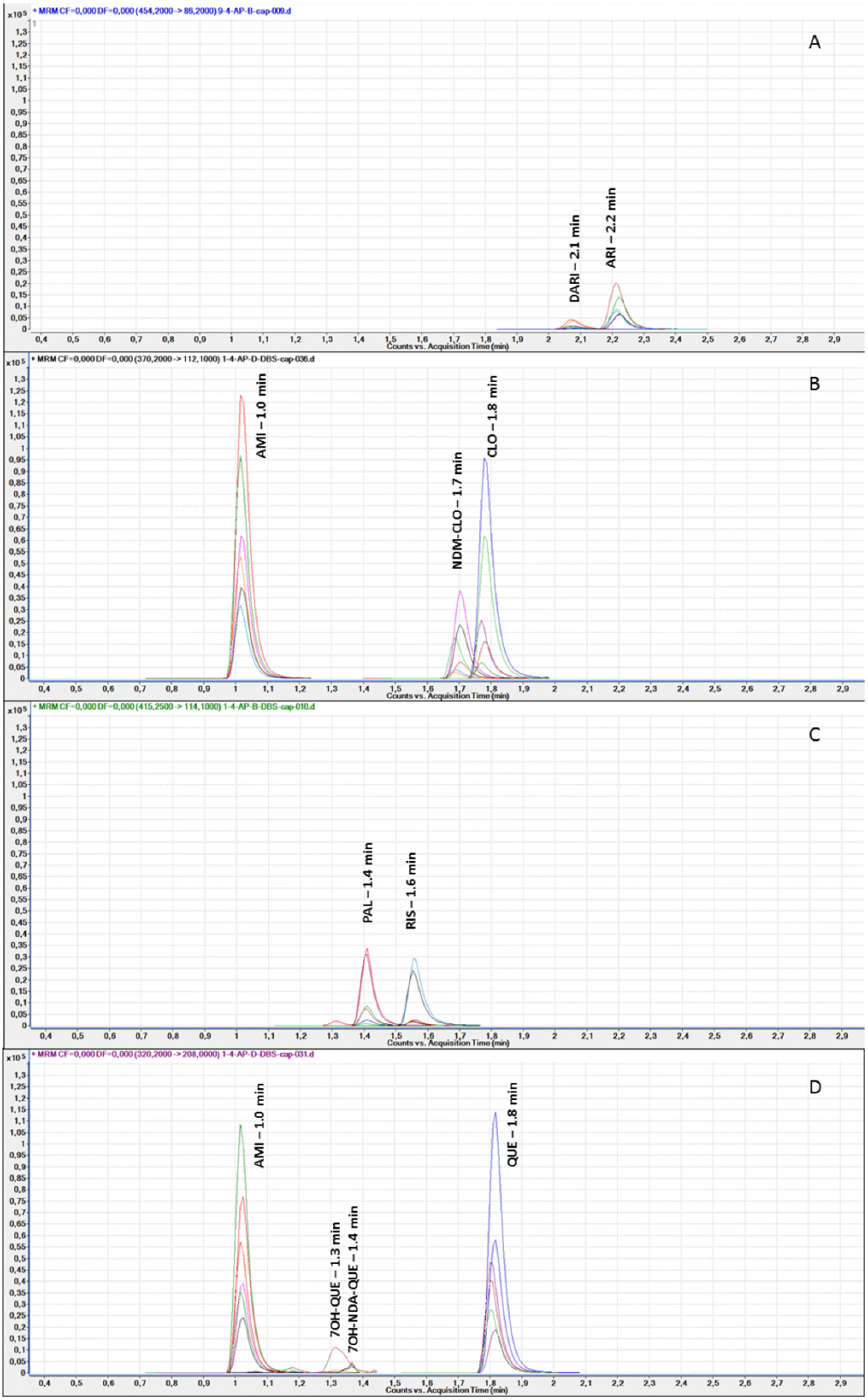

A
DARI – 2.1 min
ARI – 2.2 min
B
AMI – 1.0 min
NDM-CLO – 1.7 min
CLO – 1.8 min
C
PAL – 1.4 min
RIS – 1.6 min
D
AMI – 1.0 min
7OH-QUE – 1.3 min
7OH-NDA-QUE – 1.4 min
QUE – 1.8 min
Counts vs. Acquisition Time (min)

Table 1: Dried blood spot concentrations of calibration standards and quality control samples of all analytes

Analyte	Abbreviation	Calibrations standards (ng/ml)							Internal quality control samples (ng/ml)		
		L1	L2	L3	L4	L5	L6	L7	QC low	QC med	QC high
Haloperidol	HAL	0.5	1	2.5	5	10	25	50	1.5	7.5	35
Reduced haloperidol	RHAL	0.5	1	2.5	5	10	25	50	1.5	7.5	35
Iloperidone	ILO	0.5	1	2.5	5	10	25	50	1.5	7.5	35
Hydroxy iloperidone	HILO	0.5	1	2.5	5	10	25	50	1.5	7.5	35
Asenapine	ASE	1	2.5	5	10	25	50	100	3	20	75
Bromperidol	BRO	1	5	10	15	30	60	100	3	25	80
7-hydroxy quetiapine	7OH-QUE	1	5	10	15	30	60	100	3	25	80
7-hydroxy N-desalkyl quetiapine	7OH-NDA-QUE	1	5	10	15	30	60	100	3	25	80
N-demethylolanzapine	NDM-OLA	1	5	10	15	30	60	100	3	25	80
Risperidone	RIS	1	5	10	25	50	75	150	3	35	100
Zuclopenthixol	ZUC	1	5	10	50	100	150	300	3	75	225
Paliperidone	PAL	1	10	25	50	100	150	300	3	75	225
Olanzapine	OLA	1	10	25	50	100	150	300	3	75	225
Sertindole	SER	5	10	50	100	150	200	400	15	125	300
Lurasidone	LUR	5	25	50	100	250	500	1000	15	200	750
Pipamperone	PIP	10	50	150	300	500	750	1000	30	400	850
Dehydro-aripiprazole	DARI	10	50	100	300	500	750	1000	30	350	850
Amisulpride	AMI	10	50	100	150	300	600	1200	30	250	900
N-demethylclozapine	NDM-CLO	10	50	100	200	500	750	1500	30	350	1150
Quetiapine	QUE	10	50	250	500	750	1000	1500	30	650	1250
Aripiprazole	ARI	20	50	250	500	750	1000	1500	60	650	1250
Clozapine	CLO	50	100	250	500	750	1000	1500	150	650	1250
(Levo)sulpiride	LSUL	50	100	250	500	750	1000	1500	150	650	1250

Table 2: Accuracy and precision data for all analytes at four concentration levels.

Analyte	LLOQ			QC low			QC mid			QC high		
	Precision intraday CV (%)	Precision interday CV (%)	Accuracy bias (%)	Precision intraday CV (%)	Precision interday CV (%)	Accuracy bias (%)	Precision intraday CV (%)	Precision interday CV (%)	Accuracy bias (%)	Precision intraday CV (%)	Precision interday CV (%)	Accuracy bias (%)
7OH quetiapine	7.77	7.77	8.25	8.00	8.00	-8.75	4.55	5.78	-2.1	7.07	7.76	0.80
7OH-N-desalkyl quetiapine	9.29	12.01	16.13	6.75	8.04	-3.04	11.04	11.05	-5.75	10.47	11.48	12.8
Amisulpride	3.53	3.95	1.00	3.72	4.40	1.54	5.40	5.40	-5.78	4.91	4.95	3.76
Aripiprazole	4.01	4.65	4.75	3.93	4.88	-0.69	7.50	7.50	-5.89	2.94	4.09	-1.61
Asenapine	9.24	10.75	11.63	6.37	7.22	-5.25	8.06	8.37	-5.81	2.98	3.87	-2.07
Bromperidol	2.95	5.89	-2.50	4.80	5.20	-1.96	7.70	7.70	-1.80	2.55	3.60	-0.73
Clozapine	6.01	6.46	-2.60	3.85	4.06	9.47	5.39	5.39	-7.02	6.13	6.98	2.02
Dehydro-aripiprazole	5.39	5.96	10.25	3.93	4.80	6.71	5.58	5.58	-7.00	4.06	4.39	2.79
Haloperidol	6.56	7.40	2.75	6.02	6.67	1.92	11.22	11.22	-3.60	3.34	4.30	-1.25
Hydroxy-iloperidone	6.18	6.76	8.50	5.72	5.72	4.17	6.31	6.66	-6.53	2.76	4.49	-0.68
Iloperidone	10.63	10.63	6.25	5.49	5.60	3.58	4.16	4.16	-3.83	7.20	7.20	3.10
Levosulpiride	6.87	6.87	-2.47	3.10	3.21	7.29	5.75	5.75	-5.78	3.02	3.92	3.60
Lurasidone	14.24	16.23	14.70	**15.61**	**16.32**	**17.33**	**16.24**	**16.24**	-6.54	12.75	12.75	-3.64
Norclozapine	5.00	5.00	4.50	3.92	4.05	5.63	8.11	8.11	-7.01	4.35	4.35	0.32
Paliperidone	4.50	5.35	9.62	3.57	4.14	0.37	7.33	7.33	-5.75	2.36	3.18	1.79
Pipamperone	18.91	18.91	**-20.25**	6.79	7.38	**15.58**	6.69	7.18	2.41	7.77	8.12	-13.15
Quetiapine	5.03	6.42	6.88	3.47	3.77	0.71	6.46	6.46	-5.03	2.95	3.41	1.78
Reduced haloperidol	4.03	5.66	3.75	6.79	7.04	4.67	11.02	10.40	-3.97	5.03	5.03	-3.79
Risperidone	5.80	5.80	5.13	3.28	4.04	2.08	8.91	8.91	-6.93	3.60	4.30	-0.17
Sertindol	4.18	4.35	12.78	5.38	5.62	1.83	9.98	9.98	-5.08	5.90	6.20	1.10
Zuclopenthixol	7.51	8.76	-6.50	7.14	7.21	-4.50	8.71	8.71	-7.00	2.92	3.18	-3.36

Table 3: Recoveries, matrix effects and their respective 95% confidence intervals (CI) obtained with dried blood spot samples of different sources (n = 6) spiked with 'QC low' and 'QC high' concentrations. The CV of the internal standard (IS) corrected matrix effects were < 15 % for all compounds.

	Recovery (n = 6)				*Matrix effects (n=6)*				*IS corrected matrix effects (n=6)*					
	QC low		*QC high*		*QC low*		*QC high*		*QC low*			*QC high*		
Analyte	*mean (median) (%)*	*95% CI*	*mean (median) (%)*	*95% CI*	*mean (median) (%)*	*95% CI*	*mean (median) (%)*	*95% CI*	*mean (median) (%)*	*95% CI*	*CV*	*mean (median) (%)*	*95% CI*	*CV*
7-OH-N-desalkylquetiapine	33.2 (32.6)	28.0 - 38.4	28.7 (27.4)	25.4 - 32.0	8.8 (8.4)	6.9 - 10.7	19.0 (16.7)	12.1 - 25.9	100.7 (95.5)	82.8 - 118.6	8.5	125.8 (130.4)	112.7 - 138.9	5.5
7-OH-quetiapine	34.2 (33.6)	28.3 - 40.1	31.8 (31.9)	28.6 - 35.0	10.1 (10.1)	7.4 - 12.8	21.5 (18.4)	12.5 - 30.5	98.3 (100.1)	88.1 - 108.5	4.9	112.7 (112.8)	105.9 - 119.8	3.2
Amisulpride	62.3 (62.1)	61.0 - 63.6	65.0 (64.1)	60.1 - 69.9	91.4 (92.2)	86.6 - 96.2	96.4 (95.8)	91.9 - 100.9	93.2 (94.4)	89.9 - 96.5	1.6	97.5 (98.9)	91.9 - 103.1	2.7
Aripiprazole	69.6 (70.2)	65.8 - 73.4	70.7 (68.1)	65.7 - 75.7	53.3 (52.3)	44.6 - 62.0	65.3 (67.0)	60.4 - 70.2	93.5 (93.8)	90.7 - 96.3	1.4	97.2 (98.9)	91.1 - 103.3	3.0
Asenapine	61.8 (51.8)	44.7 - 78.9	63.4 (62.5)	54.5 - 72.3	60.5 (63.9)	54.2 - 66.8	59.4 (59.8)	55.9 - 62.9	99.2 (103.9)	85.4 - 113.0	6.6	97.3 (94.4)	90.3 - 105.3	3.4
Bromperidol	68.1 (69.7)	64.0 - 72.2	68.0 (67.8)	63.7 - 72.3	57.2 (57.3)	51.6 - 62.8	66.9 (66.1)	61.2 - 72.6	97.5 (98.0)	91.8 - 103.2	2.7	107.2 (109.6)	101.2 - 113.2	2.8
Clozapine	54.3 (54.0)	52.3 - 56.3	58.8 (58.0)	55.1 - 62.5	52.4 (51.1)	46.9 - 57.9	74.4 (72.2)	68.2 - 80.6	95.8 (95.8)	92.8 - 98.8	1.4	98.4 (99.6)	93.2 - 103.6	2.5
Dehydro-aripiprazole	63.6 (64.2)	58.6 - 68.6	70.1 (70.2)	66.4 - 73.8	39.3 (37.9)	33.4 - 45.2	52.5 (53.5)	48.8 - 56.2	100.8 (101.5)	96.1 - 105.5	2.2	103.6 (103.9)	98.3 - 108.9	2.5
Haloperidol	72.3 (73.2)	66.7 - 77.9	70.9 (68.0)	65.3 - 76.5	57.3 (58.3)	54.5 - 60.1	68.8 (67.4)	62.8 - 74.8	97.9 (98.2)	93.7 - 102.1	2.0	110.4 (114.2)	102.0 - 118.8	3.8
Hydroxy-iloperidone	68.3 (68.3)	66.7 - 69.9	68.1 (68.7)	63.4 - 72.8	84.5 (84.9)	79.9 - 89.1	100.4 (97.5)	93.3 - 107.5	92.6 (91.8)	87.6 - 97.6	2.5	98.3 (97.8)	91.5 - 105.1	3.2
Iloperidone	69.3 (69.8)	64.7 - 73.9	71.0 (69.9)	65.4 - 76.6	64.7 (64.9)	62.2 - 67.2	77.2 (74.8)	71.0 - 83.4	101.9 (101.1)	99.6 - 104.2	1.1	111.6 (112.3)	104.9 - 118.3	3.0
Levosulpiride	68.9 (69.4)	66.5 - 71.3	69.9 (68.7)	64.1 - 75.7	91.7 (92.4)	89.5 - 93.8	90.6 (90.2)	88.1 - 93.1	93.5 (93.5)	91.1 - 95.9	1.2	91.7 (93.2)	85.9 - 97.5	2.9
Lurasidone	44.6 (47.0)	39.2 - 50.0	58.5 (58.6)	54.9 - 62.1	58.4 (59.2)	49.6 - 67.2	62.8 (59.5)	50.3 - 75.3	104.1 (104.8)	95.4 - 112.8	4.1	96.7 (97.2)	87.2 - 106.2	4.6
N-desmethyl-clozapine	48.4 (48.0)	46.8 - 50.0	49.5 (49.6)	46.6 - 52.4	42.0 (40.6)	36.1 - 47.9	73.3 (70.2)	67.1 - 79.5	95.0 (95.5)	92.4 - 97.6	1.3	105.9 (105.8)	99.8 - 112.0	2.8
Paliperidone	65.0 (63.3)	60.9 - 69.1	65.0 (63.1)	61.1 - 68.9	82.3 (82.5)	78.0 - 86.6	96.3 (94.5)	91.6 - 101.0	97.1 (97.5)	95.5 - 98.7	0.8	103.7 (104.5)	97.9 - 109.5	2.7
Pipamperone	64.0 (63.3)	62.2 - 65.8	84.5 (84.9)	81.6 - 87.4	85.8 (86.4)	80.2 - 91.4	95.3 (95.3)	90.5 - 100.1	99.1 (100.0)	96.8 - 101.4	1.1	108.4 (109.5)	102.9 - 114.1	2.5
Quetiapine	70.2 (70.5)	67.8 - 72.6	72.8 (72.2)	69.3 - 76.3	87.2 (87.6)	81.2 - 93.2	97.9 (97.0)	93.9 - 101.9	94.3 (94.4)	91.9 - 96.7	1.2	97.7 (98.4)	92.7 - 103.7	2.4
Reduced haloperidol	74.7 (75.4)	68.9 - 80.5	66.4 (65.6)	58.5 - 74.3	83.8 (83.8)	79.2 - 88.4	98.1 (98.6)	93.6 - 102.6	86.2 (86.3)	84.2 - 88.2	1.0	111.1 (114.4)	102.4 - 119.8	3.9
Risperidone	67.8 (67.4)	66.4 - 69.2	68.5 (66.7)	64.7 - 72.3	89.5 (89.2)	84.7 - 94.3	97.6 (95.9)	93.0 - 102.2	96.6 (97.0)	93.9 - 99.3	1.3	103.4 (105.3)	97.7 - 109.1	2.7
Sertindole	66.4 (66.0)	60.5 - 72.3	65.2 (65.5)	61.5 - 68.9	55.3 (54.3)	45.4 - 65.2	46.0 (48.2)	40.8 - 51.2	104.0 (104.5)	100.6 - 107.4	1.6	103.7 (105.3)	96.9 - 110.5	3.2
Zuclopenthixol	62.8 (63.5)	57.1 - 68.5	69.9 (69.5)	63.7 - 76.1	46.6 (47.1)	36.6 - 56.6	42.5 (41.7)	33.7 - 51.3	102.8 (100.8)	97.4 - 108.2	2.6	103.2 (104.3)	93.5 - 112.9	4.6

** IS used for bromperidol: haloperidol-d4; IS used for levosulpiride: amisulpride-d5*

Table 4 : Stability of the analytes in dried blood spots at QC low (n=3) and QC high (n=3) after storage at room temperature, 4°C and -18°C for 1 month. Stability was calculated as deviation from samples analyzed directly after drying for 3 h at room temperature.

	QC low (mean %)			QC high (mean %)		
Analyte	1 m at RT	1 m at 4°C	1 m at -18°C	1 m at RT	1 m at 4°C	1 m at -18°C
7-OH-N-desalkylquetiapine*	102.1	92.7	97.3	125.5	104.7	99.6
7-OH-quetiapine	109.4	94.4	106.2	115.9	92.7	100.6
Amisulpride	117.7	118.7	116.3	116.7	113.8	119.3
Aripiprazole	109.0	109.9	110.0	104.4	110.3	112.1
Asenapine	106.3	99.3	90.7	108.0	109.2	109.4
Bromperidol	111.6	102.7	97.9	108.0	104.6	106.6
Clozapine	98.5	96.7	100.0	95.8	90.5	93.5
Dehydro-aripiprazole	108.1	107.1	105.6	113.8	112.0	113.0
Haloperidol	109.8	110.4	110.5	105.1	108.2	112.1
Hydroxy-iloperidone	115.5	113.1	108.9	110.0	112.7	121.5
Iloperidone	109.5	107.2	113.5	110.3	112.5	113.5
Levosulpiride	111.9	114.1	113.2	119.0	125.1	118.8
Lurasidone	106.9	106.4	107.1	101.1	108.3	107.0
N-desmethyl-clozapine	104.1	100.8	107.7	103.9	100.6	111.1
Paliperidone	110.3	107.8	112.3	116.5	118.0	119.0
Pipamperone	104.9	106.4	105.9	100.3	105.1	102.2
Quetiapine	109.1	116.5	113.2	109.4	119.0	111.9
Reduced haloperidol	103.3	106.0	112.5	103.5	112.2	111.9
Risperidone	110.9	110.7	112.5	105.7	112.5	107.2
Sertindole	104.0	115.2	114.5	109.7	112.2	109.3
Zuclopenthixol	114.4	107.9	110.4	109.4	107.6	112.4

* stability calculated as deviation from QC low after1 day

Table 5: Serum, whole blood and capillary DBS concentrations of 10 antipsychotics found in samples of 10 different patients.

Antipsychotic	Serum concentration (ng/ml)	Whole blood concentration (ng/ml)	Capillary DBS concentration (ng/ml)
Amisulpride	213.0	229.2	269.8
Aripiprazole	363.8	247.0	264.1
Bromperidol	4.1	4.4	4.7
Clozapine	484.5	467.1	456.8
Haloperidol	1.8	1.1	2.0
Paliperidone	24.8	18.4	20.9
Pipamperone	136.7	156.2	244.3
Quetiapine	85.2	57.9	59.8
Risperidone	19.4	12.3	11.6
Zuclopenthixol	4.3	4.5	4.6

Supplemental Data Table 1: Mass spectrometric conditions of all analytes including MRM transitions, collision energy (CE), qualifier/quantifier ratio, fragmentor voltage (FV), retention time (RT) used for UHPLC-MS/MS.

Analyte	Precursor ion (m/z)	Product ion (m/z)	CE (V)	Ratio (%)	FV (V)	RT (min)
Amisulpride	370.2	242.0	26	100.0	188	1.0
		196.0	42	51.2		
		112.1	22	34.4		
Amisulpride-d5	375.2	242.0	26	100.0	188	1.0
		196.0	42	51.2		
		117.1	26	33.1		
Aripiprazole	448.2	285.1	22	100.0	228	2.1
		98.1	38	44.3		
		176.1	30	41.8		
Aripiprazole-d8	456.2	293.1	26	100.0	220	2.1
		176.0	30	41.6		
		102.1	42	34.5		
Dehydro-aripiprazole	446.1	285.1	18	100.0	176	2.0
		98.1	42	34.5		
		84.1	62	4.5		
Dehydro-aripiprazole-d8	454.2	293.1	22	100.0	214	2.0
		102.1	46	33.2		
		86.2	66	5.2		
Asenapine	286.1	166.0	34	100.0	172	1.9
		229.0	18	100.6		
		215.0	30	63.4		
Asenapine-^{13}C,d3	290.1	229.0	22	100.0	172	1.9
		166.0	34	128.6		
		215.0	30	64.0		
Bromperidol*	420.1	165.0	22	100.0	172	2.0
		123.0	46	74.6		

		402.0	14	8.0		
Clozapine	327.1	270.0	18	100.0	172	1.7
		192.0	46	75.4		
		164.0	90	21.9		
Clozapine-d8	335.2	275.1	22	100.0	172	1.7
		192.0	50	80.4		
		164.0	90	35.2		
N-desmethylclozapine	313.1	192.0	42	100.0	172	1.6
		270.0	22	57.3		
		227.0	26	17.2		
N-desmethylclozapine-d8	321.2	192.0	46	100.0	172	1.6
		275.1	22	27.6		
		227.0	30	13.8		
Haloperidol	376.2	165.0	22	100.0	172	1.9
		123.0	42	122.1		
		95.1	82	53.3		
Haloperidol-d4	380.2	165.0	22	100.0	172	1.9
		123.0	42	113.2		
		95.1	82	48.2		
Reduced haloperidol	378.2	149.0	26	100.0	166	1.7
		109.0	58	61.4		
		342.1	18	11.7		
Reduced haloperidol-d4	382.2	149.0	26	100.0	166	1.7
		109.0	54	61.4		
		346.1	22	12.1		
Iloperidone	427.2	261.1	26	100.0	196	1.9
		190.0	42	83.4		
		233.1	30	76.1		
Iloperidone-d3	430.2	261.1	26	100.0	196	1.9
		190.0	42	80.0		
		233.1	30	73.6		

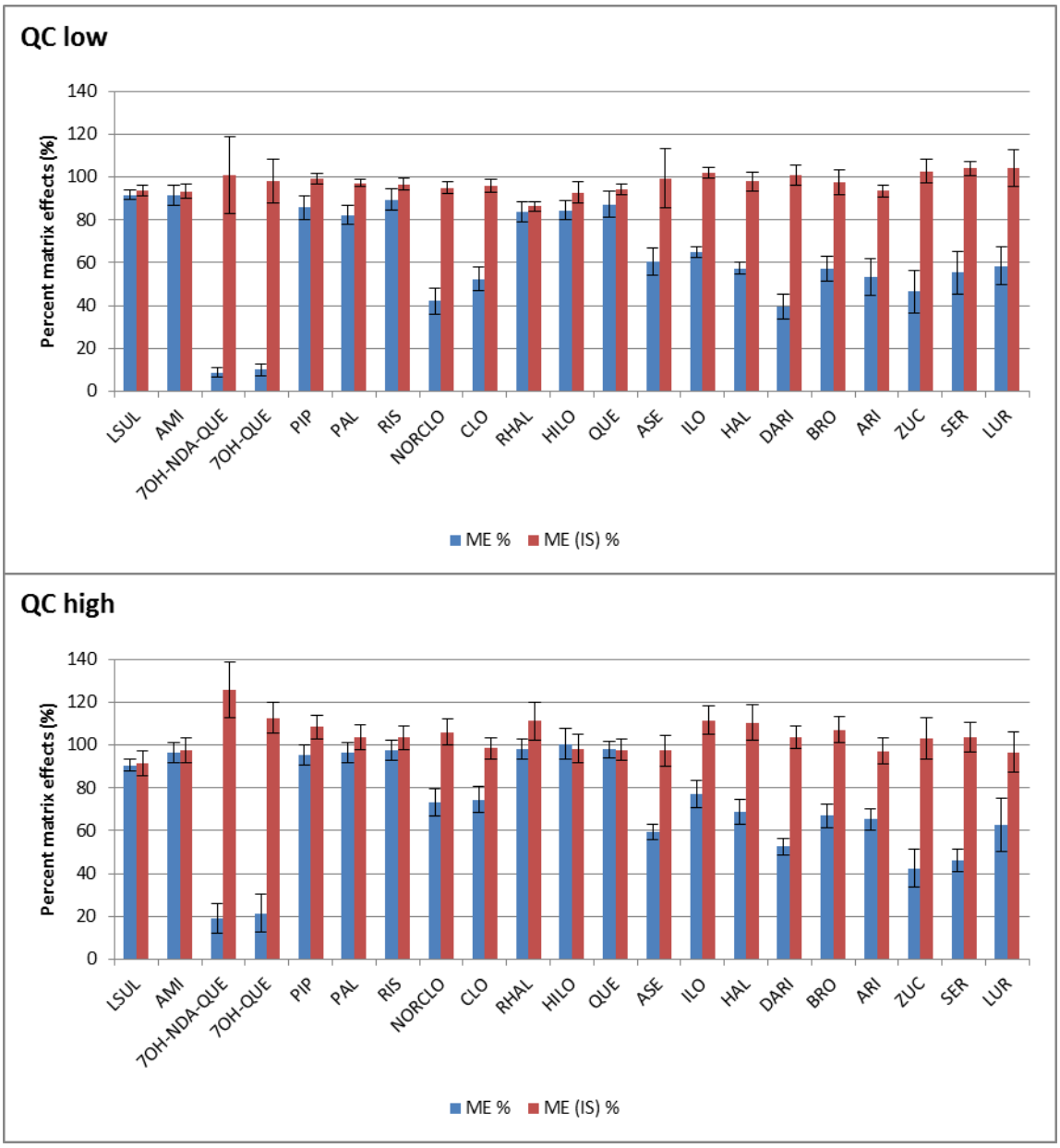
Hydroxy-iloperidone	429.2	261.1	18	100.0	196	1.7
		190.0	42	33.2		
		233.1	30	30.2		
Hydroxy-iloperidone-d4	433.3	261.1	18	100.0	196	1.7
		190.0	42	45.8		
		233.1	30	33.9		
Lurasidone	493.3	166.1	42	100.0	260	2.7
		120.1	66	40.2		
		177.0	46	35.3		
Lurasidone-d8	501.3	166.1	46	100.0	260	2.7
		120.1	66	41.9		
		181.6	46	4.8		
Levosulpiride*	342.2	112.1	22	100.0	188	0.5
		110.1	42	30.0		
		214.0	30	20.0		
Olanzapine	313.2	256.0	18	100.0	176	0.9
		198.0	42	28.0		
		169.0	42	14.4		
Olanzapine-d3	316.2	256.0	18	100.0	176	0.9
		198.0	42	27.7		
		169.0	46	15.8		
N-desmethylolanzapine	299.1	198.0	38	100.0	176	0.8
		256.0	22	83.5		
		213.0	26	63.3		
N-desmethylolanzapine-d8	307.2	198.0	38	100.0	176	0.8
		213.0	26	56.0		
		169.0	42	40.5		
Paliperidone	427.2	207.1	26	100.0	176	1.4
		110.0	46	26.2		
		82.1	58	7.3		
Paliperidone-d4	431.2	211.1	26	100.0	176	1.4

		114.1	46	24.8		
		179.0	46	3.0		
Pipamperone	376.2	165.0	26	100.0	166	1.3
		123.0	50	69.6		
		291.1	14	35.9		
Pipamperone-d10	386.3	165.0	26	100.0	166	1.2
		123.0	54	67.8		
		291.1	14	40.5		
Quetiapine	384.2	253.0	18	100.0	172	1.8
		221.1	38	52.0		
		279.1	22	15.8		
Quetiapine-d8	392.2	226.1	38	100.0	172	1.8
		257.7	22	69.2		
		286.1	22	46.7		
7-hydroxy quetiapine	400.2	269.0	18	100.0	172	1.1
		237.1	42	20.9		
		295.0	22	14.2		
7-hydroxy quetiapine-d8	408.2	274.1	22	100.0	196	1.1
		302.1	26	25.9		
		241.1	42	24.6		
7-hydroxy N-desalkyl quetiapine	312.1	226.0	26	100.0	172	1.2
		164.0	62	98.5		
		208.0	38	72.5		
7-hydroxy N-desalkyl quetiapine-d8	320.2	226.0	26	100.0	172	1.2
		164.0	62	79.7		
		208.0	42	45.0		
Risperidone	411.2	191.1	26	100.0	188	1.5
		82.1	66	8.3		
		110.0	54	7.3		
Risperidone-d4	415.3	195.1	26	100.0	188	1.5
		73.2	66	7.4		

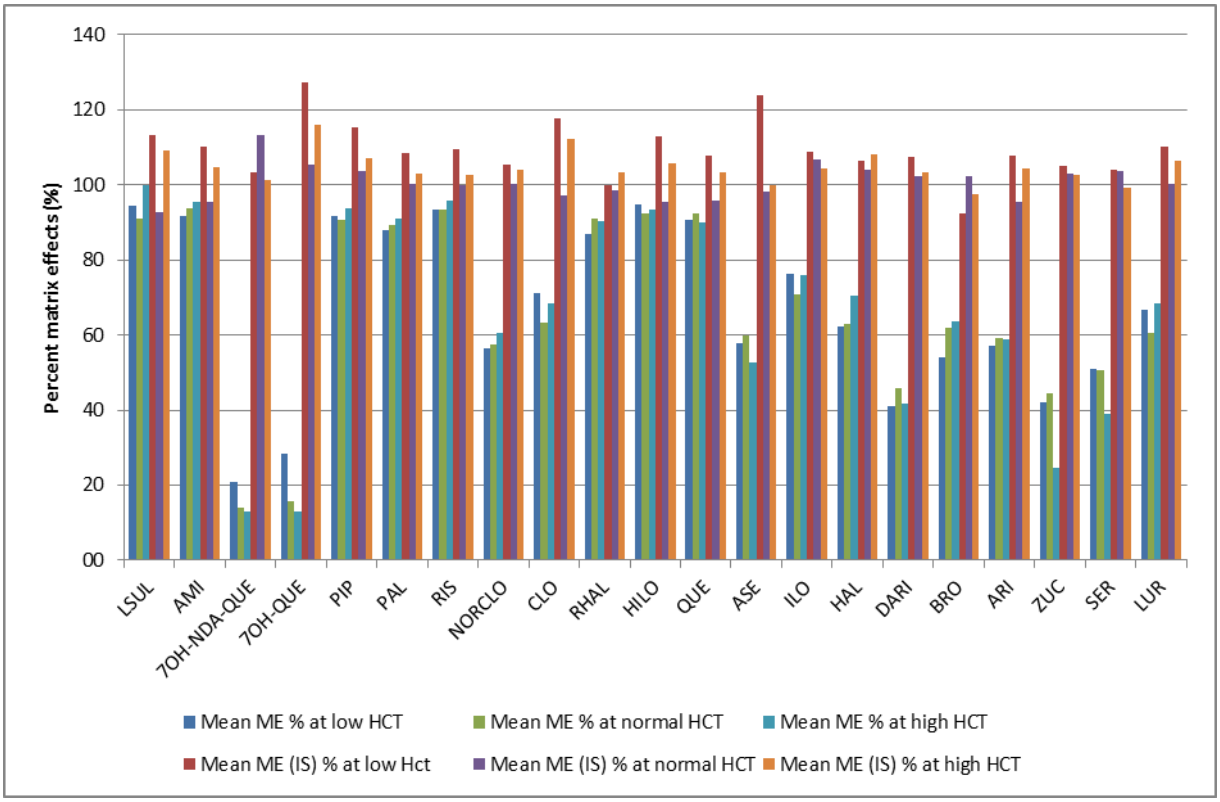
		114.1	54	6.8		
Sertindole	441.2	113.1	30	100.0	188	2.4
		71.2	54	13.6		
Sertindole-d4	445.2	117.1	34	100.0	188	2.4
		73.2	58	15.1		
Zuclopenthixol	401.2	230.9	38	100.0	188	2.4
		221.0	58	94.2		
		169.0	42	82.8		
Zuclopenthixol-d4	405.2	221.0	58	100.0	188	2.4
		231.0	34	94.9		
		104.1	26	76.8		

** IS used for bromperidol: haloperidol-d4; IS used for levosulpiride: amisulpride-d5*

Supplemental data Figure 1: Left: Mean absolute ME and mean IS corrected ME obtained by extraction of dried blood spots containing blank whole blood spiked with QC low (n = 6). Right: Mean absolute ME and mean IS corrected ME obtained by extraction of dried blood spots containing blank whole blood spiked with QC high (n = 6). The vertical bars represent the 95 % confidence interval. Analytes in the x-axis are sorted based on the elution sequence.



Supplemental data Figure 2: Absolute and IS corrected ME obtained by extraction of dried blood spots containing blank whole blood with low (19.2 %), normal (range 36.0-50.0 %) and high hematocrit (67.0 %) spiked with QC low and QC high.


Percent matrix effects (%)
140
120
100
80
60
40
20
00
LSUL
AMI
7OH-NDA-QUE
7OH-QUE
PIP
PAL
RIS
NORCLO
CLO
RHAL
HILO
QUE
ASE
ILO
HAL
DARI
BRO
ARI
ZUC
SER
LUR
Mean ME % at low HCT
Mean ME % at normal HCT
Mean ME % at high HCT
Mean ME (IS) % at low Hct
Mean ME (IS) % at normal HCT
Mean ME (IS) % at high HCT